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Interference of outer membrane protein PalA with protective immunity against *Actinobacillus pleuropneumoniae* infections in vaccinated pigs

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Abstract

The role of antibodies to the outer membrane protein PalA of *Actinobacillus pleuropneumoniae* in protective immunity was studied in pigs vaccinated with purified PalA alone and PalA in combination with toxoids of the RTX toxins ApxI and ApxII using an established challenge model with the virulent serotype 1 of *A. pleuropneumoniae*. Pigs that developed antibody titers against PalA after immunization were more significantly affected by challenge with *A. pleuropneumoniae* serotype 1. Following challenge, pigs that were immunized with PalA showed more severe respiratory symptoms, had a higher mortality rate and died faster. They also displayed much more severe lung lesions after necropsy than animals not immunized with PalA. Pigs that were immunized with toxoids of the two cytotoxins ApxI and ApxII were protected against challenge with *A. pleuropneumoniae*. In contrast, the protective efficacy of the ApxI and ApxII vaccine was completely lost when it was supplemented with PalA. Hence, antibodies induced against the outer membrane protein PalA of *A. pleuropneumoniae* aggravated the consequences of infection and counteracted the protective effect of anti-ApxI and anti-ApxII antibodies. Due to the high similarity between protein analogues of PalA from various bacteria of the *Pasteurellaceae* family such as P6 of *Haemophilus influenzae* or 16 kDa Omp of *Pasteurella multocida*, this deleterious effect of PalA in vaccination should be taken into consideration in the development of vaccines against infections with other *Pasteurellaceae*.

Keywords: Outer membrane protein; Experimental infection; Antigenicity; Immunity; Vaccine; Porcine pleuropneumonia; *Pasteurellaceae*

1. Introduction

Actinobacillus pleuropneumoniae, a Gram-negative bacterium of the family *Pasteurellaceae*, is the etiological agent of porcine pleuropneumonia, a severe contagious disease of swine with worldwide prevalence [1]. To date, fifteen serotypes have been described which variously express three different cytotoxins belonging to the RTX toxin family: ApxI, ApxII, ApxIII [2]. These toxins mainly determine the virulence of the different serotypes [3]. A fourth RTX-toxin, ApxIV, which is expressed by all serotypes of *A. pleuropneumoniae*, was recently detected and shown to be produced during infection but not during culture in growth medium [4,5]. Serotypes 1, 5a/b, 9 and 11, which express ApxI, ApxII and also ApxIV, are particularly virulent, while the other serotypes which are devoid of ApxI are generally less virulent [3]. Vaccination is an effective strategy for the prevention of porcine pleuropneumonia outbreaks. Recently, a new generation subunit vaccine, composed of the three major RTX exotoxins (ApxI, ApxII and ApxIII) and a 42 kDa outer membrane protein of *A. pleuropneumoniae*, has been developed and shown to give high protection against all 12 major serotypes (serotypes 1–12) under experimental conditions [6,7] as well as in field trials [8–10]. Vaccination of pigs with the RTX toxins alone protects against mortality but generally fails to reduce the typical *A. pleuropneumoniae* lung lesions, while the combination of RTX toxins with the 42 kDa outer membrane protein (42 kDa OMP) induced complete protection with regard to mortality as well as lung lesions [6]. The 42 kDa OMP is therefore a valuable component of the vaccine. Among the OMPs of *A. pleuropneumoniae*, PalA is the most immuno-prédominant antigen [11,12]. PalA is a 14 kDa protein, encoded by *palA* as a precursor peptide which is processed by signal sequence peptidase II and sorted by a peptide located signal

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for the final localization of the mature protein in the outer membrane. PalA is conserved in all *A. pleuropneumoniae* serotypes and induces a strong IgG response in pigs naturally or experimentally infected with *A. pleuropneumoniae* [11]. PalA shows high amino-acid (aa) sequence homology to the family of peptidoglycan associated proteins (PAL) of Gram-negative bacteria showing most similarity to the P6 protein of *Haemophilus influenzae* [11,13,14] and to the 16 kDa Omp of *Pasteurella multocida* [15]. There is evidence that PAL proteins of *Pasteurellaceae*, in particular P6 of *H. influenzae*, can act as protective antigens. P6 was therefore suggested to be included in vaccines against *H. influenzae* induced meningitis and avian cholera [15,16]. Antiserum directed against recombinant P6 protein was shown to be bactericidal against clinical *H. influenzae* isolates, including highly pathogenic non-typable strains [17]. Antibodies to P6 give passive protection to infant rats against *H. influenzae* type b-induced meningitis. In the view of the importance of PAL proteins as potential vaccine antigens, we have designed the present study to analyze the role of PalA alone, and in combination with toxoids of the RTX toxins ApxI and ApxII in induction of protective immunity against challenge of pigs with a virulent *A. pleuropneumoniae* serotype 1 strain, 4074^T.

2. Materials and methods

2.1. Bacterial strains, growth conditions and vectors

A. pleuropneumoniae 4074^T (serotype 1 reference strain) used for PCR amplification of the *palA* gene, and *A. pleuropneumoniae* serotype 1 strain 1-L-452 used for challenge, were grown on solid Columbia broth agar (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 10 mM β-NAD (Sigma Chemicals, St. Louis, MO, USA) or in liquid medium consisting of Columbia broth supplemented with 10 mM β-NAD. In order to avoid appearance of contaminating bacteria during re-isolation of *A. pleuropneumoniae* from the challenged pigs, lungs and tonsils were first superficially decontaminated with a hot spatula before sampling with sterile tools from the inside of the tissues.

Escherichia coli K-12 strain XL1-blue MRF′ (Stratagene, La Jolla, CA, USA) and *E. coli* B strain BL21 (DE3) (Novagen, Madison, WI, USA) were grown in Luria-Bertani (LB) broth [18] at 37 °C in orbital shaker incubator. Ampicillin 100 μg/ml was added when needed for selection or stabilization of plasmids. Cloning vector pETHIS-1 [4] was used for the production of recombinant poly-histidine tailed peptides.

2.2. DNA extraction, manipulation, cloning and sequence analysis

Genomic DNA from *A. pleuropneumoniae* was extracted by the guanidiumthiocyanate method [19]. Ligation, gene cloning, plasmid extraction, restriction endonuclease digestion and analysis of the DNA fragments by agarose gel electrophoresis were performed using standard protocols [18]. Plasmid extraction was done using the alkaline lysis method with the Miniprep kit (Qiagen AG, Basel, Switzerland). DNA sequencing reactions were performed with approximately 500 ng plasmid DNA per reaction mixture and 5 pmol of primer. Sequences were determined with an ABI Prism model 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequences were assembled and edited by using the Sequencher 3.0 program (Gene Codes Corp., Ann Arbor, MI, USA) to obtain contiguous sequences.

2.3. Production of antigens for vaccines

PalA protein from *A. pleuropneumoniae* serotype 2 strain S411 for vaccination was produced as described earlier and involved heat extraction in PBS buffer (Na-phosphate 50 mM, pH 7.5, NaCl 0.85%) for 1 h at 60 °C, ammonium sulfate fractionation and separation by SDS-PAGE followed by electroelution [11]. The commercially available subunit vaccine, Porcilis App™ (Intervet International, Boxmeer, the Netherlands), was used as reference preparation for the titration of the ApxI and ApxII antigens. ApxI and ApxII proteins were prepared from supernatants of *A. pleuropneumoniae* serotype 5b bacteria grown at 37 °C for 6 h in Columbia broth supplemented with βNAD 10 μg/ml and $CaCl_2$ 25 mM. Cells were removed by centrifugation and sterile filtration through a low protein binding 0.45 μm filter (Acrocap, #4482, Gelman Laboratory, Ann Arbor, MI, USA). ApxI and ApxII were subsequently concentrated by ultrafiltration on a 500 kDa MW cut-off polyether sulfon filter (Amicon, bioseparations, Millipore, Bedford, MA, USA). Protein concentrations were measured by the method of Bradford [20]. ApxI and ApxII preparations were analyzed by standard SDS-PAGE stained with Coomassie blue [21] where they revealed a predominant band at 105 kDa indicating that ApxI and ApxII represented the major proteins (estimated to 90% of the total proteins) in the preparations [22].

For the vaccination of pigs, the following vaccines were produced: (i) Vaccine I (PalA) consisted of 80 μg PalA protein in 2 ml Diluvac Forte® adjuvant formulation (Intervet International) per vaccine dose with 0.02% (v/v) formaldehyde final concentration. (ii) Vaccine II (PalA + ApxI + ApxII) consisted of 80 μg recombinant PalA, ApxI and ApxII in 2 ml Diluvac Forte® adjuvant formulation per vaccine dose with 0.02% (v/v) formaldehyde final concentration. (iii) Vaccine III (ApxI + ApxII) consisted of ApxI and ApxII in 2 ml Diluvac Forte® adjuvant formulation per vaccine dose with 0.02% (v/v) formaldehyde final concentration; the concentrations of ApxI and ApxII in vaccines II and III were titrated by specific antigenic mass ELISA and adjusted to the same concentrations of antigens in the commercially available vaccine Porcilis App™ corresponding to approximately 50 μg of ApxI and 25 μg of ApxII per dose

(2 ml) of vaccine. (iv) Vaccine IV (Porcilis App™) was a positive protection control, using the commercially available subunit vaccine Porcilis App™ (Intervet International).

2.4. Vaccinations and challenge

Vaccination was done intramuscularly. For each vaccine, three 6 weeks old SPF landrace pigs were immunized on day 0 (zero) and subsequently received a booster vaccination on day 28. Three control animals (group V) received injections of adjutants on the same days. Two weeks after booster vaccination, on day 42, control sera were taken and then the pigs were challenged with *A. pleuropneumoniae* serotype 1 strain 1-L-452. The bacteria for challenge were grown for 6 h and washed twice by centrifugation at 10,000 × *g* for 15 min and re-suspension in the original volume of PBS buffer. Pigs of all groups were exposed at the same time for 15 min to the bacteria in an aerosol using a De Vilbiss nebulizer [23]. After challenge, fever and respiratory symptoms were recorded. Two weeks later, on day 56, the pigs were slaughtered, unless they died of the infection or had to be euthanized prior to this date. Blood was taken on the day of immunization, on day 42 (prior to challenge) and from the surviving animals at day 56. Dead and euthanized animals were subjected to post-mortem examination for typical lung lesions and recovery of challenge bacteria from lungs and tonsils.

2.5. Production of recombinant PalA'His

In order to specifically monitor the antigenic response to PalA in vaccinated pigs, we have produced an antigenically specific recombinant peptide consisting of the hydrophilic central- and C-terminal part of PalA containing the 106 most C-terminal amino acids (11.54 kDa) fused to 6 N-terminal and 10 C-terminal histidine residues. The corresponding part of the *palA* gene was amplified using the oligonucleotide primers X89009-L (cgc*catatg*-CAAACTCGTTACACCACT) and X89009-R (cgc*ggatcc*-GTATTCTAATACTGCACG). The primers were designed to contain recognition sites for the restriction enzymes *Nde*I and *Bam*HI (shown in italic letters) by the addition of supplementary nucleotides (shown in lower case). This procedure allowed the PCR amplification product to be cloned into the *Nde*I and *Bam*HI cloning sites of vector pETHIS-1. PCR was carried out with a DNA thermal cycler (GeneAmp 9600; Perkin-Elmer Cetus, Norwalk, CT, USA) in a 50 μl reaction mixture containing 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween 20, 170 μM of each dNTP, 0.25 μM forward and reverse primers, 0.5 units *Pwo* polymerase and 5 ng genomic DNA of *A. pleuropneumoniae* strain 4074. The amplification was carried out for 35 cycles (30 s denaturation at 94 °C, 30 s annealing at 50 °C, 1 min elongation at 72 °C). The PCR product was purified using the QIA quick PCR purification kit (Qiagen, Basel, Switzerland), cut with the restriction enzymes *Nde*I and *Bam*HI and ligated to *Nde*I and *Bam*HI digested pETHIS-1. Ligated DNA was transformed into *E. coli* strain XL1-blue MRF and positive clones were selected by colony hybridization using a digoxigenin labelled DNA probe for *palA* [11]. A positive clone, named pJFF-PalA1H, was sequenced to verify the integrity of the cloned segment of *palA* and the fusions with the poly-His codons of pETHIS-1. For biosynthesis of recombinant PalA'His peptide, plasmid pJFFPalA1H was introduced into *E. coli* BL21 (DE3) for expression, which was induced by addition of 1 mM IPTG at mid-exponential phase and incubation for a further 2.5 h. Following induction, the poly-histidine tailed fusion protein PalA'His was purified from cell extracts dissolved with 6 M guanidine hydrochloride using Ni^{2+} chelate affinity chromatography (Qiagen) according to the manufacturer's instructions. The bound PalA'His was eluted by slowly decreasing the pH from 8.0 to 4.5 with 50 mM potassium phosphate buffer, 300 mM NaCl, 6 M guanidine hydrochloride. Following elution at pH 4.5, the fusion protein was dialyzed against 50 mM phosphate buffer, 300 mM NaCl, pH 7.5. The fractions were analyzed by standard SDS-PAGE [18] and protein concentrations were measured by the method of Bradford [20].

2.6. Serological assays

In order to monitor the immune response to PalA in vaccinated pigs, we have developed an immunoblot assay using purified recombinant PalA'His as an antigen. Samples of 100 μg PalA'His were separated on 14% SDS-PAGE of 8 cm × 8 cm and subsequently blotted onto nitrocellulose membranes (BioRad, Hercules, CA, USA, product no. 162-0112). The dried membranes were cut into 20 strips with a width of 4 mm in order to get immunoblot strips each containing 5 μg of PalA'His. The strips were reacted with pig sera diluted 1:500, using the standard immunoblot procedure [18]. Phosphatase labelled goat antibodies, directed against pig IgG (Kirkegaard & Perry, Gaithersburg, MD, USA; product no. 051401), diluted 1:2000, followed by addition of nitroblue tetrazolium and bromochlorindolyl phosphate in alkaline phosphate buffer [18], were used to visualize bound antibodies. Each lot of immunoblot strips was controlled using rabbit anti-PalA antibodies [11] at a dilution of 1:1000 and phosphatase labelled goat antibodies directed against rabbit IgG (Kirkegaard & Perry, product no. 075-1506) diluted 1:2000.

Specific antibody titers in serum against ApxI, ApxII, ApxIII and 42 kDa OMP were determined by indirect ELISA as described [24]. This ELISA is based on the antigens ApxI, ApxII, ApxIII and 42 kDa OMP that were purified from *A. pleuropneumoniae* strains with serotypes not related to the production strains used for vaccine antigen production, in order to avoid possible cross-reaction with contaminating polysaccharides. Concentrations of antigen preparations were determined in antigenic mass ELISA (toxins) or SDS-PAGE (OMP), relative to reference

preparations. The antigenic mass ELISA determines the concentration in arbitrary units relative to the reference preparations. The concentrations of the proteins used for coating of the micro-titer plates for antibody ELISA were in the order of 1 μg/ml. Final adjustments were made depending on antibody titers obtained with positive and negative reference sera. Routinely samples were measured twice and the mean values were reported. The differences of the two measurements were below 10%.

3. Results

3.1. Immune response to PalA in vaccinated pigs

The results of the analysis of anti-PalA antibodies in the blood sera by immunoblots with PalA'His are shown in Fig. 1. All pigs were free of anti-PalA antibodies before vaccination. Pigs vaccinated with vaccines I or II, which contained PalA alone or in combination with ApxI + ApxII, showed a weak reaction 28 days after the first vaccination, and a strong anti-PalA reaction 2 weeks after the booster vaccination. One pig belonging to group II (#176), from which a blood sample was taken before slaughter, showed an anti-PalA reaction on day 56 (Fig. 1). Groups III and IV of pigs that were vaccinated with vaccine containing ApxI + ApxII or with the commercial vaccine respectively, did not show anti-PalA antibodies after first vaccination (day 28) or after booster vaccination (day 42). One pig (#181) of group III showed a very weak anti-PalA reaction 2 weeks after challenge on day 56, which is thought to be due to the challenge with *A. pleuropneumoniae* (Fig. 1).

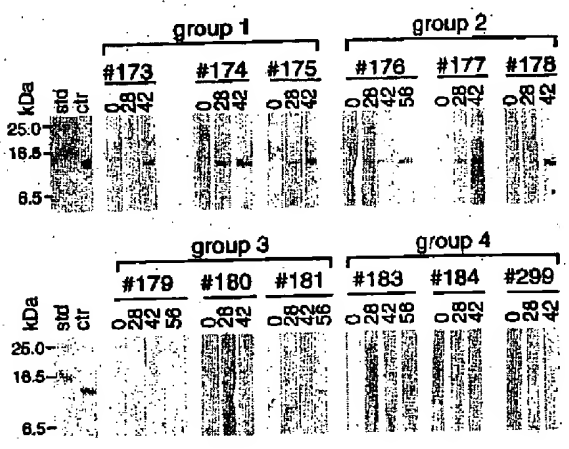

Fig. 1. Serological analysis of anti-PalA antibodies. Immunoblot strips each containing 5 μg PalA'His protein incubated with the different sera before and after vaccination are shown. Figures in the horizontal position indicate the pig numbers, figures in the vertical position indicate days after vaccination. Group I included pigs #173, #174 and #175 vaccinated with vaccine I (PalA); group II included pigs #176, #177 and #178 vaccinated with vaccine II (PalA + ApxI + ApxII); group III included pigs #179, #180 and #181 vaccinated with vaccine III (ApxI + ApxII); group IV included pigs #183, #184 and #299 vaccinated with vaccine IV (Porcilis App™, (Intervet) containing ApxI, ApxII, ApxIII and 42 kDa Omp). Note that pigs #173, #174, #175 and #177 died or were euthanised before the end of the experiment. Std: broad range pre-stained protein markers (New England Biolabs, Beverly, Mass, USA; no. 77085); the position of the molecular masses of 25, 16.5 and 6.5 kDa are indicated.

Table 1
Antibody responses after two vaccinations, measured 2 weeks after booster vaccination and before challenge

Vaccine (content)	Antibody titer against antigen			
	ApxI	ApxII	ApxIII	42 kDa OMP
I (PalA)	<2[a]	<2[a]	<2.0[a]	<2.0[a]
II (PalA + ApxI + ApxII)	3.3	2.6	<2.0[a]	<2.0[a]
III (ApxI + ApxII)	3.5	2.6	<2.0[a]	<2.0[a]
IV (Porcilis App™)	3.2	2.8	2.8	2.4
V (Control)	<2.0[a]	<2.0[a]	<2.0[a]	<2.0[a]

ELISA titers are expressed as the logarithm ($\log_{10}$) of the reciprocal of the highest dilution of serum with an OD above that of the preimmune serum for each pig diluted 1:100 as defined by [24]. Figures represent the mean values of two measurements.

[a] Indicates below detection level.

The antibody responses to vaccination against ApxI, ApxII, ApxIII and 42 kDa OMP were as expected for the various vaccines containing these antigens, as shown in Table 1. The comparison of antibody titers against ApxI and ApxII after vaccination with PalA (group II) or without PalA (group III) added, showed that the serological titers of ApxI and ApxII were not affected by the presence of PalA in the vaccine.

3.2. Protection against infection with *A. pleuropneumoniae*

The susceptibility of the pigs to *A. pleuropneumoniae* was assessed by challenge of a non-vaccinated group of pigs. In this group, all pigs showed high fever after the challenge as well as abdominal respiration and coughing, which are typical signs for pleuropneumonia (Table 2). One of the three pigs died 6 days post-infection. Upon necropsy, all pigs showed lung lesions affecting, on average, 50–75% of the lungs. These results showed that the challenge with *A. pleuropneumoniae* serotype 1 strain resulted in typical signs of porcine pleuropneumonia under the given experimental conditions.

The group of pigs vaccinated with the commercial vaccine Porcilis App™ (vaccine IV) showed significantly fewer clinical signs after challenge with low or no fever and virtually no respiratory distress. No mortality occurred in this group and no or only minor lung lesions affecting less than 25% of the lung were detected. These results testify to the high protection obtained with the subunit vaccine.

In the group of pigs vaccinated with ApxI and ApxII alone (vaccine III), a high level of protection against infection with *A. pleuropneumoniae* serotype 1 was observed. This was similar to the group vaccinated with the commercial subunit

Table 2
Effect of challenge with *A. pleuropneumoniae* serotype 1 on pigs vaccinated with various vaccines

Vaccine (content)	Pig no.	Fever[a]	Respiratory distress[b]	Mortality (day p.i.)[c]	Lung lesion score[d]	Re-isolation[e]	
						Lung	Tonsil
I (PalA)	173	nd	3	+ (1)	4	+	+
	174	nd	3	+ (1)	4	+	−
	175	2	3	+ (2)	4	+	Cont.
	avg	2	3	3/3 (1.3)	4	3/3	1/2
II (PalA + ApxI + ApxII)	176	2	2	−	2	+	+
	177	0	3	+ (2)	4	Cont.	Cont.
	178	2	2	−	3	+	+
	avg	1.3	2.3	1/3 (2)	3	2/2	2/2
III (ApxI + ApxII)	179	0	0	−	0	−	+
	180	0	0	−	0	−	+
	181	1	1	−	0.5	+	−
	avg	0.3	0.3	0/3	0.2	1/3	2/3
IV (Porcilis App™)	299	1	1	−	1	+	−
	183	0	0	−	0	−	+
	184	1	0	−	1	+	−
	avg	0.7	0.3	0/3	0.6	2/3	1/3
V (Control)	185	2	2	+ (6)	4	+	+
	186	2	2	−	2	−	+
	189	2	2	−	3	+	+
	avg	2	2	1/3 (6)	3	2/3	3/3

[a] Scale used: (0) no fever (<40 °C); (1) fever (>40–41 °C); (2) high fever (>41 °C); (nd) could not be determined due to the rapid death of the animal.
[b] Scale used: (0) no distress, normal respiration; (1) increased respiration rate; (2) abdominal respiration and/or coughing; (3) dyspnea.
[c] (+) Indicates dead pig or pig euthanised because of severe clinical signs (number in brackets gives the day of death post-infection); (−) indicates that the pig did not die.
[d] Scale used: (0) no lesions; (1) 1–25% of the lungs affected; (2) 26–50% affected; (3) 51–75% affected; (4) 76–100% affected (severe lesions).
[e] (+) Indicates animal from which the challenge strain was re-isolated; Cont.: isolation of *A. pleuropneumoniae* not possible since culture was contaminated by other bacteria; (−) indicates that no *A. pleuropneumoniae* could be isolated.

vaccine. This group showed no mortality, only one pig with mild fever and increased respiration rate and virtually no lung lesions after necropsy.

Vaccine II, containing PalA added to ApxI and ApxII, showed no protection. On average, the pigs showed clinical signs similar to the non-vaccinated control group. In this group, the mortality rate was similar to that of the control group. In addition, lung lesions in this group showed the same scores as in the non-vaccinated group.

Vaccination of pigs with purified PalA alone (vaccine I) showed no protection, but rather severe symptoms and rapid death after challenge with *A. pleuropneumoniae* serotype 1. All three pigs died within 1 or 2 days after challenge and subsequent necropsy revealed severe lung lesions. In comparison with the non-vaccinated control group, vaccination of pigs with PalA alone resulted in a significant increase in mortality, three out of three PalA vaccinated animals died, compared to one animal out of three that died from the control group ($\chi^2 = 3.0$; $P = 0.08$) (Table 2). Most significant, was the sudden death after challenge of the PalA vaccinated pigs one to 2 days post-infection, while in the control group one pig died 6 days post-infection (Table 2). Upon necropsy, all pigs vaccinated with PalA showed very severe lung lesions (score 4 with 75–100% of the lungs affected) compared to one animal out of three in the control group (Table 2).

From pigs of all groups, the challenge strain could be re-isolated after necropsy from lungs and from tonsils, showing no differences between the control group and the different vaccinated groups. From a few animals, re-isolation of the challenge strain was hindered due to strong growth of other bacteria on the culture media (Table 2).

4. Discussion

The family of PAL proteins has been characterized as proteins constituting integral parts of the outer membrane of many Gram-negative bacteria. They are highly conserved within given bacterial species. Moreover, they show strong similarities between different bacterial species. They are described as very strongly antigenic proteins in several pathogenic bacteria such as *A. pleuropneumoniae* [11], *H. influenzae* [25,26], *Legionella pneumophila* [27], *P. multocida* [15], *Campylobacter jejuni* [28] and *Brucella abortus* [29]. The prototype of PAL, the peptidoglycan associated lipoprotein PAL of *E. coli* was shown to form complexes,

one type together with the outer membrane proteins TolA, TolQ and TolR, and a second type with the periplasmic protein TolB, in order to maintain the outer membrane integrity by anchoring the outer membrane to the peptidoglycan layer [30]. Mutants deficient in the PAL protein appear to be debilitated for growth under certain conditions. A PAL-deficient mutant of *Haemophilus ducrei* was shown to display a reduced capacity for pustule formation compared to its wild type parent when injected in human volunteers [31]. In addition, the mutant was more susceptible to the antibiotics Erythromycin, Cefotaxime and Ciprofloxacin than the wild type strain and could not be reisolated from pustules in contrast to the wild type strain [31]. The PAL proteins seemed therefore to be valuable targets for immune protection. Several reports of indirect evidence indicate that protein P6 (alternatively named Hi-PAL) of *H. influenzae* is involved in the induction of protective immunity against *H. influenzae* infections. They include studies showing that antibodies to P6 are protective in the infant rat model against invasive *H. influenzae* type b [32]; the demonstration of bactericidal activity for *H. influenzae* of an antibody to P6 immunopurified from human serum [33]; and the fact that rabbit antiserum raised to purified P6 is bactericidal for a broad range of *H. influenzae* strains including many non-typable *H. influenzae* [16]. From these results it was thought that P6 might be a valuable subunit in vaccines against *H. influenzae* infections. Moreover, PAL of other pathogens were considered as appropriate antigens in vaccines. However, no protection against avian cholera was obtained by vaccination with recombinant P6-like protein from *P. multocida* [34].

In our approach to examine the effect of a PAL protein as candidate for a vaccine, we have taken advantage of a well established challenge model of pigs with *A. pleuropneumoniae* to test the efficacy of PalA alone or PalA in combination with known protective antigens as vaccines against porcine pleuropneumonia. In our study, a small number of animals were tested for ethical reasons and, therefore, it does not allow for thorough statistical analysis. However, our data clearly show that pigs, which developed antibody titers against PalA after immunization, showed more significant symptoms, a much higher mortality and died much faster after challenge with *A. pleuropneumoniae* than unvaccinated control pigs. The more severe lung lesions found after necropsy in the PalA vaccinated group further highlighted this observation. Hence the higher mortality and the faster occurring death in the PalA vaccinated group seemed to be an aggravation of the pleuropneumonia and was not due to secondary effects like septic shock. The difference in protective efficacy between the vaccine containing ApxI and ApxII and the vaccine with ApxI, ApxII and PalA is of particular interest. The pigs vaccinated with the two cytotoxins ApxI and ApxII were well protected against challenge with *A. pleuropneumoniae* serotype 1, like the group that was vaccinated with the commercially available subunit vaccine Porcilis App™. In contrast, the protective efficacy of ApxI and ApxII vaccine was completely lost when it was supplemented with PalA, as shown in the group of pigs vaccinated with vaccine II. Hence, PalA antibodies significantly reduce the protective effect of anti-ApxI and anti-ApxII antibodies. The mechanism behind this negative effect of PalA on protective immunity is not known. However, we rule out the possibility that PalA would have had a negative effect on the induction of antibodies against ApxI and ApxII, as the anti ApxI and ApxII titers are the same in the presence or absence of PalA. Since PalA is well conserved in all serotypes of *A. pleuropneumoniae*, the effect is expected to occur with any of the serotypes.

Although the limited number of animals used did not allow us to perform dose-dependence studies, we conclude that PalA should be absent in vaccines against *A. pleuropneumoniae*. Our study does not permit us to extrapolate whether other PAL antigens such as P6 of *H. influenzae* would yield similar effects. However, PalA shows very high similarity to P6 of *H. influenzae* (73% identical and 82% similar aa) and to P6-like protein of *P. multocida* (72% identical and 97% similar aa). Vaccination with other PAL proteins could therefore result in similar negative effects, or as in the case of the P6-like protein of *P. multocida*, give no protection [34]. When using whole cell preparations of bacterial cultures (bacterin vaccines), it must be noted that the concentration of PAL proteins varies depending on the mode of cultivation and preparation of the bacteria, and might therefore vary from one batch to another. Consequently, this could be an explanation of the variations in protective efficacy of certain bacterin vaccines, which are currently observed. Since the negative effect of PAL proteins in vaccines seems to be non-predictable, it would be advisable to avoid PAL proteins in vaccines unless specific evidence for a positive effect on protection is found. The existence of such negatively acting components gives further support to the need for development of well defined subunit vaccines against bacterial infections.

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